



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : C07K 7/10, 13/00, A61K 45/05 A61K 37/02</p>	<p>A1</p>	<p>(11) International Publication Number: WO 93/1703 (43) International Publication Date: 2 September 1993 (02.09.93)</p>
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<p>(54) Title: METHODS OF TREATING DIABETES</p> <p>(57) Abstract</p> <p>A method of treating a patient with diabetes involving administering to the patient a hybrid molecule which contains a cytotoxin covalently joined to interleukin-2 which is capable of binding to interleukin-2 receptor on a cell that contributes to the disease state of the cell and decreasing the viability of that cell.</p>		

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METHODS OF TREATING DIABETESBackground of the Invention

The field of the invention is diabetes.

5 Diabetes mellitus is a prevalent and degenerative disease characterized by insulin deficiency, which prevents normal regulation of blood glucose levels leading to hyperglycemia and ketoacidosis.

Insulin promotes glucose utilization, protein
10 synthesis, formation and storage of neutral lipids, and the growth of some cell types. Insulin is produced by the β cells within the islets of Langerhans of the pancreas.

Some individuals with diabetes are not dependent
15 upon the administration of exogenous insulin, other individuals are completely dependent upon exogenously administered insulin. Insulin-dependence is related to the degree of destruction of the β islet cells. Diabetic patients that are not insulin-dependent can be diagnosed
20 as having diabetes if they exhibit some of the symptoms of the disease, e.g., hyperglycemia, and have antibodies to insulin or islet cells, or both. Such patients may progress to full-blown insulin-dependent diabetes mellitus if they are not treated.

25 Insulin-dependent diabetes mellitus (IDDM) is a T cell dependent autoimmune disease. Activated T cells selectively target insulin producing beta cells and mediate their destruction. In the most severe form of diabetes, the autoimmune reaction causes complete
30 destruction of β cells, resulting in an absolute lack of insulin production in the individual.

The importance of T cells in human diabetogenic autoimmunity is emphasized by the ability of cyclosporine A to cause remission in new onset IDDM (Stiller et al.,

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1984, Science 223:1362). However, cyclosporin A-induced remissions have not been proven to be permanent, and the chronically administered high doses of cyclosporin A required to maintain a remission are associated with
5 nephrotoxicity. Thus, cyclosporin A is an unlikely candidate for general clinical use (Drash et al., 1990, in Pediatric Clinics of North America: Current Issues in Pediatric and Adolescent Endocrinology 37:6).

Studies in the non-obese diabetic mouse (NOD)
10 indicate that the disease in mice is similar to IDDM in humans (Makino et al., 1980, Exp. Anim. 29:1). Anti-T cell monoclonal antibodies (anti-Thy 1.2 or anti-CD4) prevent disease in NOD mice (Harada and Makino, 1986, Exp. Anim. 35:539; Shizura et al., 1988, Science 250:659)
15 and anti-CD25 has been shown to prevent insulinitis in NOD mice (Kelley et al., 1988, J. Immunol. 140:59). However, the action of anti-CD25 antibody was subsequently shown to be blocked by anti-idiotypic antibodies which had been generated in NOD mice (Pankewycz et al., 1988, J.
20 Autoimmunity 1:119). T cell clones obtained from the islets of prediabetic mice with insulinitis precipitate diabetes when transferred into prediabetic NOD mice (Pankewycz et al., 1991, Eur. J. Immunol. 21:873; Haskins et al., 1989, Proc. Natl. Acad. Sci. 86:8000).

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Summary of the Invention

The invention features a method of treating a patient with diabetes which involves administering to the patient a hybrid molecule which contains a cytotoxin covalently fused to interleukin-2, or a receptor-binding
30 portion thereof. The molecule is capable of binding to a cell which contributes to the disease state of the patient and contains a high affinity interleukin-2 receptor. The molecule is further capable of decreasing

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the viability of the cell, preferably by killing the cell.

The cytotoxin molecule is preferably a fragment of a peptide toxin which is enzymatically active but which does not possess generalized eukaryotic receptor binding activity.

Preferably, the fragment of peptide toxin can be fragment A of diphtheria toxin and enough of fragment B of diphtheria toxin to form a pore in a cell membrane.

10 The hybrid molecule is most preferably DAB₄₈₆IL-2 or DAB₃₈₉IL-2.

The term diabetes includes patients that are insulin-dependent, that are not dependent on exogenously administered insulin but that exhibit some of the symptoms of diabetes, for example, hyperglycemia, and that have antibodies to either islet cells, insulin, or both. Patients that are not insulin-dependent eventually become dependent as the disease progresses. Accordingly, the invention features methods for the treatment of patients with each of the levels of diabetes as defined above.

The invention also features a method of treating a patient with diabetes wherein there is administered a hybrid cytotoxin IL-2 molecule and cyclosporin A. Cyclosporin A is administered either in conjunction with the hybrid molecule, or is administered after the diabetic condition of the patient has substantially improved as a result of treatment with the hybrid molecule. In this method of treatment, cyclosporin A can be administered at dosage levels that are effective and yet non-toxic compared to the levels required to treat a patient that does not receive the hybrid molecule.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

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Detailed Description

The drawings will first briefly be described.

Drawings

Figure 1 is a graphical representation of the effect of DAB₄₈₆IL-2 and DA(197)B₄₈₆IL-2 on the acquisition of diabetes. Prediabetic NOD mice were irradiated (1,000 rad) and injected i.v. with 2×10^7 mononuclear spleen cells from diabetic NOD mice. a) Mice were injected daily with vehicle buffer (n=8), 10 μ g of DA(197)B₄₈₆IL-2 (n=8) or 10 μ g of DAB₄₈₆IL-2 (n=9) during 4 weeks after adoptive transfer. b) Mice were injected daily with vehicle buffer (n=8), or 5 μ g of DAB₄₈₆IL-2 (n=8) during 4 weeks after adoptive transfer.

Figure 2 is a graphical representation of the effect of DAB₄₈₆IL-2 on the acquisition of diabetes. Prediabetic NOD mice were irradiated (1,000 rad) and injected i.v. with 2×10^7 mononuclear spleen cells from a diabetic mouse. Mice were treated with vehicle buffer (TBS), 10, 5 or 1 μ g/day of DAB₄₈₆IL-2.

20 Interleukin-2 as a Targeting Agent

Interleukin-2 (IL-2) or any IL-2 receptor binding derivative thereof can be used as a targeting agent for a cytotoxin. The DNA and amino acid sequences of IL-2 are known (Tadatsugu et al., Nature 302:305, 1983), and its structure has been predicted by x-ray crystallography (Brandhuber et al., Science 238:1707, 1987). Analysis of genetically engineered variants of IL-2 has provided some information concerning which residues are important for IL-2R binding (Collins et al., Proc. Natl. Acad. Sci. USA 85:7709, 1988) and bioactivity (Cohen et al. Science 234:349, 1989; Collins et al., supra). Variants of IL-2 which are useful in the invention include deletion mutants (Genbauffe et al., USSN 388,557, hereby

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incorporated by reference) which lack one or more amino acid residues in the region between residue 74 and residue 79 (numbering according to Williams et al., Nucl. Acids Res. 16:1045, 1988). These mutants effectively target toxins to IL-2R-bearing cells (Genbauffe et al., supra). Generally, IL-2 variants useful for targeting a cytotoxin must efficiently bind IL-2R and be endocytosed. The ability of various derivatives to bind to the IL-2 receptor can be tested with an IL-2R binding assay described below.

In designing molecules targeted to cells bearing the IL-2 receptor it must be recognized that the IL-2 receptor, like other receptors, has several forms; and it may be desirable to target cells bearing one form and not another. The human interleukin-2 receptor has a high-, an intermediate-, and a low-affinity form. The high affinity receptor has an apparent K_d of $\sim 10^{-10}M$ and is composed of two subunits, p55 and p75 (also called p70). When expressed on the cell surface, both the p75 and p55 subunits are capable of binding IL-2. The p75 subunit corresponds to the intermediate affinity receptor ($K_d \sim 8.2 \times 10^{-10}M$), and p55 subunit corresponds to the low affinity receptor ($K_d \sim 1-3 \times 10^{-8}M$). The p75 subunit is expressed on the surface of resting T cells, natural killer cells, monocytes/macrophages, and lymphokine-activated killer (LAK) cell precursors, while the high affinity receptor is expressed on activated T- and B-cells.

In the method of the invention it may be desirable to target only cells bearing the high affinity receptor. In these circumstances, useful molecules will eliminate or neutralize cells bearing the high affinity IL-2 receptor at a concentration which leaves cells bearing the intermediate or low affinity receptor largely unaffected. When the molecule, like IL-2 itself, has

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affinity for all three classes of IL-2 receptor, selectivity can be accomplished by administering the molecule at a concentration which does not permit significant binding to cells bearing lower affinity receptors. A hybrid molecule may have altered receptor affinities compared to IL-2. Such hybrid molecules may be more or less selective for cells bearing the high affinity IL-2 receptor. For example, cells bearing the high-affinity receptor are 500-1000 times more sensitive to DAB₄₈₆IL-2, a fusion protein consisting of part of diphtheria toxin and part of IL-2, than are cells bearing the intermediate-affinity receptor (Waters et al., Eur. J. Immunol. 20:785, 1990).

A cytotoxin can be attached to an IL-2 derivative in a number of ways. Preferably, an IL-2/toxin hybrid is a hybrid protein produced by the expression of a fused gene. Alternatively, the cytotoxin and the IL-2 derivative can be produced separately and later coupled by means of a non-peptide covalent bond. Linkage methods are described below.

Useful cytotoxins are preferably significantly cytotoxic only when present intracellularly and are substantially excluded from any given cell in the absence of a targeting domain. Peptide toxins fulfill both of these criteria and are readily incorporated into hybrid molecules. A mixed cytotoxin, a cytotoxin composed of all or part of two or more toxins, can also be used. Several useful toxins are described in more detail below.

Toxins

The toxin molecules useful in the method of the invention are preferably toxins, such as peptide toxins, which are significantly cytotoxic only when present intracellularly. Of course, under these circumstances the molecule must be able to enter a cell bearing the targeted receptor. This ability depends on the nature of

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the molecule and the nature of the cell receptor. For example, cell receptors which naturally allow uptake of a ligand are likely to provide a means for a molecule which includes a toxin to enter a cell bearing that receptor.

5 The peptide toxin useful in the methods of the invention is fused to an IL-2R binding domain by producing a recombinant DNA molecule which encodes a hybrid protein molecule. Such an approach ensures consistency of composition.

10 Many peptide toxins have a generalized eukaryotic receptor binding domain; in these instances the toxin must be modified to prevent intoxication of non-receptor bearing cells. Any such modifications must be made in a manner which preserves the cytotoxic functions of the
15 molecule (see U.S. Department of Health and Human Services, U.S. Serial No. 401,412). Potentially useful toxins include, but are not limited to: cholera toxin, ricin, O-Shiga-like toxin (SLT-I, SLT-II, SLT II_V), LT toxin, C3 toxin, Shiga toxin, pertussis toxin, tetanus
20 toxin, *Pseudomonas* exotoxin, alorin, saporin, modeccin, and gelatin.

Mixed Toxins

The cytotoxic portion of some molecules useful in the invention can be provided by a mixed toxin molecule.

25 A mixed toxin molecule is a molecule derived from two different polypeptide toxins. Generally, as discussed above in connection with diphtheria toxin, polypeptide toxins have, in addition to the domain responsible for generalized eukaryotic cell binding, an enzymatically
30 active domain and a translocation domain. The binding and translocation domains are required for cell recognition and toxin entry respectively. The enzymatically active domain is the domain responsible for cytotoxic activity once the molecule is inside a cell.

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Naturally-occurring proteins which are known to have a translocation domain include diphtheria toxin, *Pseudomonas* exotoxin A, and possibly other peptide toxins. The translocation domains of diphtheria toxin and *Pseudomonas* exotoxin A are well characterized (see, e.g., Hoch et al., Proc. Natl. Acad. Sci. USA 82:1692, 1985; Colombatti et al., J. Biol. Chem. 261:3030, 1986; and Deleers et al., FEBS Lett. 160:82, 1983), and the existence and location of such a domain in other molecules may be determined by methods such as those employed by Hwang et al. Cell 48:129, 1987); and Gray et al. Proc. Natl. Acad. Sci. USA 81:2645, 1984).

One useful IL-2/mixed toxin hybrid molecule is formed by fusing the enzymatically active A subunit of *E. coli* Shiga-like toxin (Calderwood et al., Proc. Natl. Acad. Sci. USA 84:4364, 1987) to the translocation domain (amino acid residues 202 through 460) of diphtheria toxin, and to IL-2. This three-part hybrid molecule, SLT-A/DTB'/IL-2, is useful in the method of the invention in the same way as DAB₄₈₆IL-2 described above. The IL-2 portion of the three-part hybrid causes the molecule to attach specifically to IL-2R-bearing cells, and the diphtheria toxin translocation portion acts to insert the enzymatically active A subunit of the Shiga-like toxin into the targeted cell. The enzymatically active portion of Shiga-like toxin, like diphtheria toxin, acts on the protein synthesis machinery of the cell to prevent protein synthesis, thus killing the cell. The difference between these two types of hybrid toxins is the nature of their enzymatic activities: the enzymatic portion of DAB₄₈₆IL-2 catalyzes the ADP-ribosylation by nicotinamide adenine dinucleotide of Elongation Factor 2, thereby inactivating this factor which is necessary for protein synthesis, while the enzymatic portion of SLT-A/DTB'/IL-2 is a rib nuclease capable of cleaving ribosomal RNA at a

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critical site, thereby inactivating the ribosome. SLT-A/DTB'/IL-2 hybrid would therefore be useful as a treatment for the same indications as DAB₄₈₆IL-2, and could be substituted or used in conjunction with it if, for example, a patient's activated T-cells develop a resistance to DAB₄₈₆IL-2.

Linkage of Toxins to Binding Ligands

The binding ligand and the cytotoxin of useful hybrid molecules can be linked in several ways. If the hybrid molecule is produced by expression of a fused gene, a peptide bond serves as the link between the cytotoxin and the binding ligand. Alternatively, the toxin and the binding ligand can be produced separately and later coupled by means of a non-peptide covalent bond.

For example, the covalent linkage may take the form of a disulfide bond. In this case, if the binding ligand is a protein, e.g., IL-2, the DNA encoding IL-2 can be engineered to contain an extra cysteine codon as described in Murphy et al. U.S. Serial No. 313,599, hereby incorporated by reference. The cysteine must be positioned so as to not interfere with the IL-2R binding activity of the molecule. For example, the cysteine codon can be inserted just upstream of the DNA encoding Pro₂ of the mature form of IL-2. The toxin molecule must be derivatized with a sulfhydryl group reactive with the cysteine the modified IL-2. In the case of a peptide toxin this can be accomplished by inserting a cysteine codon into the DNA sequence encoding the toxin. Alternatively, a sulfhydryl group, either by itself or as part of a cysteine residue, can be introduced using solid phase polypeptide techniques. For example, the introduction of sulfhydryl groups into peptides is described by Hiskey (Peptides 3:137, 1981). Derivatization can also be carried out according to the

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method described for the derivatization of a peptide hormone in Bacha et al. U.S. Patent No. 4,468,382, hereby incorporated by reference. The introduction of sulfhydryl groups into proteins is described in Maasen et al. (Eur. J. Biochem. 134:32, 1983). Once the correct sulfhydryl groups are present, the cytotoxin and IL-2R binding ligand are purified, both sulfur groups are reduced; cytotoxin and ligand are mixed; (in a ratio of about 1:5 to 1:20) and disulfide bond formation is allowed to proceed to completion (generally 20 to 30 minutes) at room temperature. The mixture is then dialyzed against phosphate buffered saline to remove unreacted ligand and toxin molecules. Sephadex chromatography or the like is then carried out to separate on the basis of size the desired toxin-ligand conjugates from toxin-toxin and ligand-ligand conjugates.

Assays for IL-2 Receptor Binding

The IL-2R binding ability of various molecules can be measured using an IL-2R assay for high affinity (Ju et al., J. Biol. Chem. 262:5723, 1987) or intermediate affinity receptors (Rob et al., Proc. Natl. Acad. Sci. USA 84:2002, 1987).

Assays for Toxicity

Molecules of the invention can be screened for the ability to decrease viability of cells bearing the targeted receptor by means of assays such as those described below.

Toxicity towards IL-2R bearing cells can be tested as follows. Cultured HUT 102/6TG (Tsuda et al., Proc. Natl. Acad. Sci. USA 83:9694, 1986) or YT2C2 (Teshigawari et al., J. Exp. Med. 165:223, 1987) cells are maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 25 mM HEPES (pH 7.4), 2mM 1-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (Hazelton, Lenexa, KS). Cells are

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seeded in 96-well V-bottomed plates (Linbro-Flow Laboratories, McLean, VA) at a concentration of 1×10^5 per well in complete medium. Putative toxins are added to varying concentrations ($10^{-12}M$ to $10^{-6}M$) and the

5 cultures are incubated for 18 hrs. at $37^\circ C$ in a 5% CO_2 atmosphere. Following incubation, the plates are centrifuged for 5 min. at $170 \times g$, and the medium removed and replaced with $100 \mu l$ leucine-free medium (MEM, Gibco) containing $8 \mu Ci/ml$ (3H -leucine; New England Nuclear,

10 Boston, MA). After an additional 90 min. at $37^\circ C$, the plates are centrifuged for 5 min. at $170 \times g$, the medium is removed, and the cells are collected on glass fiber filters using a cell harvester (Skatron, Sterling, VA). Filters are washed, dried, and counted according to

15 standard methods. Cells cultured with medium alone serve as the control.

Diphtheria Toxin-based Molecules

Diphtheria toxin can be used to produce molecules useful in the methods of the invention. Diphtheria

20 toxin, whose sequence is known, is described in detail in Murphy U.S. Patent 4,675,382, hereby incorporated by reference. The natural diphtheria toxin molecule secreted by *Corynebacterium diphtheriae* consists of several functional domains which can be characterized,

25 starting at the amino terminal end of the molecule, as enzymatically-active Fragment A (amino acids Gly₁ - Arg₁₉₃) and Fragment B (amino acids Ser₁₉₄ - Ser₅₃₅), which includes a translocation domain and a generalized cell binding domain (amino acid residues 475 through 535).

30 The process by which diphtheria toxin intoxicates sensitive eukaryotic cells involves at least the following steps: (i) the binding domain of diphtheria toxin binds to specific receptors on the surface of a sensitive cell; (ii) while bound to its receptor, the

35 toxin molecule is internalized into an endocytic vesicle;

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(iii) either prior to internalization, or within the endocytic vesicle, the toxin molecule undergoes a proteolytic cleavage between fragments A and B; (iv) as the pH of the endocytic vesicle decreases to below 6, the toxin crosses the endosomal membrane, facilitating the delivery of Fragment A into the cytosol; (v) the catalytic activity of Fragment A (i.e., the nicotinamide adenine dinucleotide - dependent adenosine diphosphate (ADP) ribosylation of the eukaryotic protein synthesis factor termed "Elongation Factor 2") causes the death of the intoxicated cell. It is apparent that a single molecule of Fragment A introduced into the cytosol is sufficient to block down the cell's protein synthesis machinery and kill the cell. The mechanism of cell killing by *Pseudomonas* exotoxin A, and possibly by certain other naturally-occurring toxins, is very similar.

DAB₄₈₆IL-2, a fusion protein in which the receptor binding domain of diphtheria toxin has been replaced by a portion of human IL-2 (Williams et al., J. Biol. Chem. 35:20673, 1990; see also Williams et al., Protein Eng. 1:493, 1987), is an example of a molecule useful in the method of the invention. This molecule selectively kills IL-2R-expressing tumor cells and lymphocytes (Waters et al., Eur. J. Immunol. 20:785, 1990; Kiyokawa et al., Cancer Res. 49:4042, 1989). Because of its ability to kill activated lymphocytes, DAB₄₈₆IL-2 has been used to control graft rejection (Pankewycz et al., Transplantation 47:318, 1989; Kickman et al., Transplantation 47:327, 1989) and to treat certain autoimmune disorders (Forte et al., 2nd International Symposium on Immunotoxins, 1990).

DAB₄₈₆IL-2 is a chimeric molecule consisting of Met followed by amino acid residues 1 through 485 of the mature diphtheria toxin fused to amino acid residues 2

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through 133 of IL-2. Thus, DAB₄₈₆IL-2 includes all of diphtheria toxin fragment A, which encodes the enzymatically active portion of the molecule, and a portion of fragment B. The portion of fragment B present
5 in DAB₄₈₆IL-2 does not include the generalized receptor binding domain but does include the translocation domain which facilitates delivery of the enzymatically active portion into the cytosol.

A second molecule useful in the invention is
10 DAB₃₈₉IL-2, which is similar to DAB₄₈₆IL-2 except that it contains 97 amino acids less than DAB₄₈₆IL-2.

Preparation of DAB₄₈₆IL-2 and DAB₃₈₉IL-2

DAB₄₈₆IL-2 and DAB₃₈₉IL-2 are produced and purified as described in U.S. Patent Application No. 07/537,430,
15 filed on June 13, 1990, and corresponding PCT Patent Application No. PCT/US91/01282, herein incorporated by reference.

IL-2 Toxin Treatment Blocks Diabetogenic Autoimmunity in NOD Mice

20 High affinity interleukin-2 receptor (IL-2R) is a feature of recently activated T cells and is not detected on resting T cells (Smith, 1988, Science 240:1169; Teshigawara et al., J. Exp. Med. 165:223). An ideal therapeutic for diabetes should rapidly and selectively
25 destroy the activated, autoaggressive T cells. It has been demonstrated that diabetogenic cells express IL-2R in vivo (Pankewycz et al., 1991, Eur. J. Immunol. 21:873). The data described below demonstrates that specific elimination of IL-2R⁺ T-cells aborts the
30 diabetogenic process. In summary, NOD mice were treated with the IL-2 fusion toxin (DAB₄₈₆IL-2). DAB₄₈₆IL-2 selectively binds to the high affinity IL-2-R heterodimer (Waters et al., 1990, Eur. J. Immunol. 20:785). This fusion toxin exerts potent immunosuppression in vivo by

IL-2 - fused to diphtheria toxin A chain
DAB₄₈₆IL-2

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- preventing delayed type hypersensitivity (Kelley et al., 1988, Proc. Natl. Acad. Sci. USA 85:3980) via selective targeting of antigen activated T-cells (Bastos et al., 1990, J. Immunol. 145:3535) and by prolonging
- 5 engraftment, often indefinitely, of allogeneic heart or islet grafts (Kirkman et al., 1989, Transplantation 47:327; Pankewycz et al., 1989, Transplantation 47:318). The data described below demonstrate that DAB₄₈₆IL-2 treatment inhibits diabetogenic autoimmunity in NOD mice.
- 10 In addition, diabetic NOD mice treated with DAB₄₈₆IL-2 do not bear spleen cells that transfer IDDM into prediabetic NOD mice.

Materials and Methods

- As described above, DAB₄₈₆IL-2 is the product of a
- 15 fusion gene in which the human IL-2 sequence replaces the codons for the receptor-binding domain of diphtheria toxin (Williams et al., 1987, Protein Eng. 1:493). DA(197)B₄₈₆IL-2, is a mutant form of DAB₄₈₆IL-2 in which a single amino acid change at position 52 (glycine to
- 20 glutamic acid) results in a loss of ADP-ribosyltransferase activity. DA(197)B₄₈₆IL-2 was used as a control molecule. These proteins were purified from cellular extracts of Escherichia coli. They were free of contamination by endotoxins, were suspended in a vehicle
- 25 buffer (Tris Buffer Saline), pH 7.9, and were aliquoted at concentrations of 10, 5 and 1 µg/ml. Recombinant human IL-2 was purchased from Biogen Inc. (Cambridge, Massachusetts).

Adoptive Transfer of Diabetes

- 30 Two month-old, female NOD mice (Brigham and Women's Hospital; original breeding pairs Jackson Laboratory, Bar Harbor Maine) were lethally irradiated (1,000 rads) and injected intravenously within 4 hours of irradiation with 1.5 to 2.0 X 10⁷ mononuclear spleen
- 35 cells harvested from spontaneously diabetic NOD mice that

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had blood glucose levels of > 300 mg/dl. Administration of this number of cells uniformly induced diabetes in 2 month old female NOD mice by 21 days.

Mice were treated subcutaneously with $10 \mu\text{g/d}$, $5 \mu\text{g/d}$, or $1 \mu\text{g/d}$, of $\text{DAB}_{486}\text{IL-2}$ or with $10 \mu\text{g/d}$ of $\text{DA}(197)\text{B}_{486}\text{IL-2}$ or with 0.1 ml of the vehicle buffer. To assess the ability of $\text{DAB}_{486}\text{IL-2}$ to prevent diabetogenic autoimmunity, some diabetic NOD were treated with $10 \mu\text{g/d}$ $\text{DAB}_{486}\text{IL-2}$ for 1 week. Spleen cells from treated or control mice were adoptively transferred to lethally irradiated 2 month old prediabetic NOD which received no further treatment.

Results

$\text{DAB}_{486}\text{IL-2}$ protects NOD mice from adoptively transferred diabetes

$\text{DAB}_{486}\text{IL-2}$ treatment administered subcutaneously prevents diabetes in pre-diabetic NOD mice adoptively transferred with mononuclear spleen cells from diabetic NOD mice. Each of 8 NOD mice injected with a vehicle buffer became diabetic (measured as a sustained blood glucose level of > 200 mg/dl, i.e., three standard deviations above the mean of the blood glucose level measured in prediabetic NOD mice) within 8 weeks of adoptive transfer. By comparison, only 1/9 mice injected with $10 \mu\text{g/d}$ of $\text{DAB}_{486}\text{IL-2}$ became diabetic ($p < 0.001$) in this period, while 4/8 of those mice injected with $\text{DA}(197)\text{B}_{486}\text{IL-2}$ remained euglycemic ($p < 0.007$) in the same period (Fig. 1a). Histologic examination of the group of mice receiving $\text{DAB}_{486}\text{IL-2}$ sacrificed at 9 weeks, who remained normoglycemic after receiving diabetogenic T cells, had minimal numbers of mononuclear infiltrates within islets (1.2 ± 0.6 , $n=8$). By comparison, 5/8 vehicle control treated mice who became diabetic by 4 weeks were already dead and there were few islets remaining in the 3 diabetic sacrificed mice.

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Unexpectedly, when DAB₄₈₆IL-2 or DAB₃₈₉IL-2 was administered intravenously to prediabetic NOD mice that had received mononuclear spleen cells from diabetic NOD mice, neither compound had any impact on the diabetic process.

In a second set of experiments designed to determine if DAB₄₈₆IL-2 targets diabetogenic cells, spleen cells transferred from diabetic donor NOD mice injected with DAB₄₈₆IL-2 were transferred into recipients and were found to be incapable of inducing diabetes within 8 weeks after adoptive transfer (0/4 mice). By comparison, spleen cells from untreated diabetic donor NOD mice induced diabetes in 8/9 animals within this same period (Table 2). Therefore, elimination of IL-2R bearing cells from diabetic donor spleen cells *in vivo* results in at least partial elimination of autoimmune diabetogenic T-cells.

A third set of experiments was designed to determine whether mice treated for 4 weeks with 5 µg/d DAB₄₈₆IL-2 would remain permanently free of IDDM. Although the rate of development of diabetes was markedly delayed in DAB₄₈₆IL-2 treated mice, the 8 treated mice eventually became diabetic between 8 and 16 weeks after adoptive transfer of diabetogenic cells. All control mice became diabetic by 5 weeks after adoptive transfer (Fig. 1b). Of interest, the level of blood glucose in the DAB₄₈₆IL-2 treated mice that eventually became diabetic was always lower than untreated mice. For example, at the onset of IDDM, untreated mice had blood glucose levels of 274 ± 8mg/dl, while treated mice had values of 231 ± 10mg/dl (p<0.01). Blood glucose levels continued to increase in untreated mice and the majority of this group achieved values above 300 mg/dl, while mice receiving either 10 or 5 µg/d DAB₄₈₆IL-2 never exhibited values as high as 300 mg/dl. Thus, even though mice

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treated with 4 weeks of DAB₄₈₆IL-2 eventually did become diabetic, treatment with this fusion-toxin dampened the severity and rate of progression of disease.

Mice treated with IL-2 alone had a similar incidence of diabetes (7/8) as compared to the vehicle control group (5/5), and mice injected with DA(197)B₄₈₆IL-2 (5/8) also became diabetic (Table 1). Thus, IL-2 alone or DA(197)B₄₈₆IL-2 could not account for the beneficial action of DAB₄₈₆IL-2.

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TABLE 1

IL-2 DOES NOT ALTER THE TEMPO OF DIABETES IN NOD MICE

<u>TREATMENT</u>	<u>INCIDENCE OF DIABETES* (%)</u>
IL-2 (2.5µg/d) n=8	88
DA(197)B ₄₈₆ IL-2(10µg/d) n=8	63
VEHICLE BUFFER n=5	100

10

* Blood glucose determination at 3 weeks after adoptive transfer of pre-diabetic NOD mice irradiated (1000 rad) and injected i.v. with 2×10^7 spleen cells from diabetic NOD mice. Subcutaneous treatments were given daily in a volume of 0.1 ml.

15

TABLE 2

PRETREATMENT OF DIABETIC NOD MICE WITH DAB486-IL-2 PROTECTS FROM ADOPTIVE TRANSFER OF DIABETES

<u>PRETREATMENT OF DONORS</u>	<u>ONSET OF DIABETES (weeks after adoptive transfer)</u>
none+ n=9	3, 3, 3, 4, 4, 5, 5, 8
DAB ₄₈₆ IL-2 (10µg/d)* n=4	8, 14, >16, >16 (p<0.01)

25 + Young NOD mice were irradiated (1,000 rad) and reconstituted with 2×10^7 mononuclear spleen cells from recently diabetic untreated NOD mice and received no further treatment.

30 * Young NOD mice were irradiated (1,000 rad) and reconstituted with 2×10^7 mononuclear spleen cells from recently diabetic NOD mice that were treated with DAB₄₈₆ IL-2 (10µg/day) for one week and received no further treatment.

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Therapy

Generally, the molecules of the invention can be administered by intravenous infusion/injection. They may also be administered subcutaneously or intramuscularly.

- 5 Dosages of molecules useful in the methods of the invention will vary, depending on factors such as the age of the patient, the severity of diabetes in the patient and the route of administration. Patients may be treated with 25 to 300 kU/kg of either DAB₄₈₆IL-2 or DAB₃₈₉IL-2.
- 10 Either compound can be administered to a patient daily, or intermittently, or daily for a period of time, followed by intermittent administration.

- More than 100 patients have received DAB₄₈₆IL-2 in Phase I/II clinical protocols. The molecule is well
- 15 tolerated with the maximum tolerated dose (MTD) established by transient asymptomatic hepatic transaminase elevations in about 30% of patients treated at the MTD. Anti-tumor effects have been seen in approximately 40% of patients; responses were noted in B-
- 20 cell leukemias and lymphomas, cutaneous T-cell lymphoma and Hodgkin's disease (LeMaistre et al., Blood 360a:abstract 1429, 1990; Woodworth et al., Fourth International Conference on Human Retrovirology, 1991). Serum concentrations of 10⁻⁸M DAB₄₈₆IL-2 have been safely
- 25 achieved in patients with IL-2 receptor expressing malignancies. Significant anti-tumor effects have been observed in highly refractory leukemia/lymphoma patients and these effects have occurred despite the presence of elevated soluble IL-2R levels in all patients. This
- 30 observation is consistent with data which suggest that soluble IL-2R does not interfere with binding of IL-2 to the high affinity interleukin-2 receptor. Animal and human studies have demonstrated that DAB₄₈₆IL-2 has no general immunosuppressive effect (LeMaistre et al.,
- 35 *supra*; Woodworth et al., *supra*).

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Experiments indicate that binding and internalization of DAB₄₈₆IL-2 by cells bearing the high affinity IL-2 receptor occurs within 30 minutes of exposure, resulting in maximal inhibition of protein synthesis within several hours. Therefore, the molecule should be effective even if the serum half-life is rather short.

Clinical Studies in Patients with IDDM

A phase I/II study of safety, tolerability, pharmacokinetics and biological response of DAB₄₈₆IL-2 in humans with recent onset IDDM is underway.

This pilot study was designed to evaluate the safety and tolerability of DAB₄₈₆IL-2 in IDDM patients and to assess pharmacokinetics and immune function effects, together with changes in diabetic status as determined by insulin requirement, C-peptide levels, and control of hyperglycemia. Based on experience in similar studies, such effects can be preliminarily assessed over a 4 to 6 week period following agent administration, and thus, onset autoimmune diabetes mellitus provides a clinical model for the evaluation of potential immunologic and anti-diabetic effects of a new therapeutic like DAB₄₈₆IL-2.

DAB₄₈₆IL-2 has been administered to individuals over 15 years of age with symptoms \leq 4 months in duration, HLA DR3 or 4 and/or anti-islet cell antibody formation. Patients received a 60 minute intravenous infusion daily for 7 days in a cohort dose-escalation protocol, evaluating dose levels of 0.025, 0.05, and 0.075 mg/kg. This pilot study has evaluated 24 patients, each receiving a single course.

To date, 18 patients are evaluable. The agent has been well tolerated in this group of patients; there has been mild transient hepatic transaminase elevations in 15 to 20% of patients, two transient episodes of edema and

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two incidences of mild rash suggestive of hypersensitivity-like effects. Surprisingly, 8 of these patients (3 in the 0.025 mg/kg, 3 in the 0.05 mg/kg, and 2 in the 0.075 mg/kg dose groups) have had a substantial decrease in insulin requirement, together with a sustained increase in C-peptide (≥ 0.6 nanomolar), and a normalization of glycosylated hemoglobin. Data analysis for the other 6 patients is underway.

Further, in these responding patients, the addition of cyclosporin A at a non-nephrotoxic dose (4-5 mg/kg/d) resulted in sustained or even greater improvement in the response parameters. Since this low dose of cyclosporin A would not be expected to induce such an improvement (Bach, 1989 in Thompson, ed. Cyclosporin A: Mode of Action and Clinical Applications. London Kleuver Academic Publishers, 181), this observation suggests that treatment of diabetic patients with an IL-2 fusion toxin such as DAB₄₈₆IL-2 followed by, or in conjunction with, low dose cyclosporin A is a highly effective way to safely administer cyclosporin A to patients with diabetes.

The data described above demonstrate that DAB₄₈₆IL-2 is capable of suppressing IDDM in NOD mice, and that this suppression can be attributed to the ability of DAB₄₈₆IL-2 to target IL-2 expressing T cells and kill them. The data described above also demonstrate that DAB₄₈₆IL-2 is surprisingly useful in reducing insulin dependence in human patients that already have IDDM.

What is claimed is:

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1. A method for preparing a medicament for treatment of diabetes, said method comprising admixing pharmaceutically acceptable carrier and hybrid molecule comprising a first and a second portion joined together covalently, said first portion comprising a cytotoxin capable of decreasing cell viability, said second portion comprising all of or an interleukin-2 receptor-binding portion of interleukin-2 capable of binding to a high affinity interleukin-2 receptor on a cell, wherein said cell contributes to a diabetic condition.

2. A method for preparing a medicament for treatment of diabetes said method comprising admixing cyclosporin A and the hybrid molecule of claim 1, said cyclosporin A being present at an effective yet substantially non-toxic level per dosage.

3. The method of claim 1 or 2 wherein said hybrid molecule kills cells bearing said receptor.

4. The method of claim 1 or 2 wherein said cytotoxin is a fragment of a peptide toxin which is enzymatically active but which does not possess generalized eukaryotic receptor binding activity.

5. The method of claim 1 or 2 wherein said cytotoxin comprises fragment A of diphtheria toxin and enough of fragment B of diphtheria toxin to form a pore in a cell membrane.

6. The method of claim 1 or 2 wherein said hybrid molecule is DAB₄₈₆IL-2.

7. The method of claim 1 or 2 wherein said hybrid molecule is DAB₃₈₉IL-2.

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8. The method of claim 6, wherein said DAB₄₈₆IL-2 is present at 25 to 300kU/kg per dosage.

9. The method of claim 7, wherein said DAB₃₈₉IL-2 is present at 25 to 300kU/kg per dosage.

5 10. The method of claim 1 or 2 wherein said diabetes is not being treated by the administration of exogenous insulin.

11. The method of claim 1 or 2 wherein said diabetes is being treated by the administration of
10 exogenous insulin.

12. Use of a hybrid molecule comprising a first and a second portion joined together covalently, said first portion comprising a cytotoxin capable of decreasing cell viability, said second portion comprising
15 all of or an interleukin-2 receptor-binding portion of interleukin-2 capable of binding to a high affinity interleukin-2 receptor on a cell, wherein said cell contributes to a diabetic condition, in the preparation of a medicament for the treatment of diabetes.

20 13. The use of cyclosporin A in the preparation of a medicament for use in conjunction with the medicament prepared as in claim 12.

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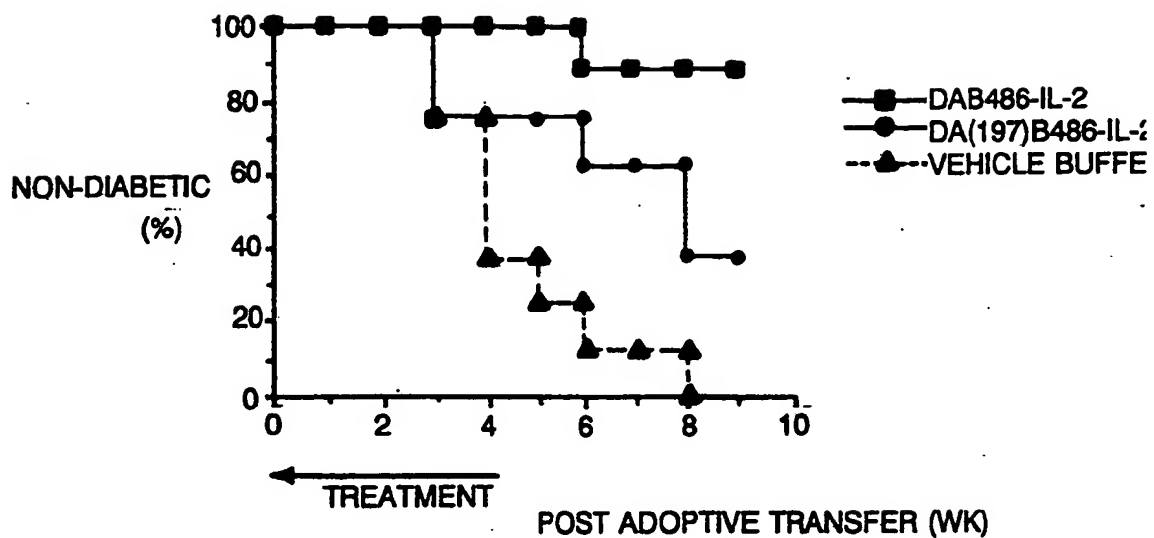


FIG. 1A

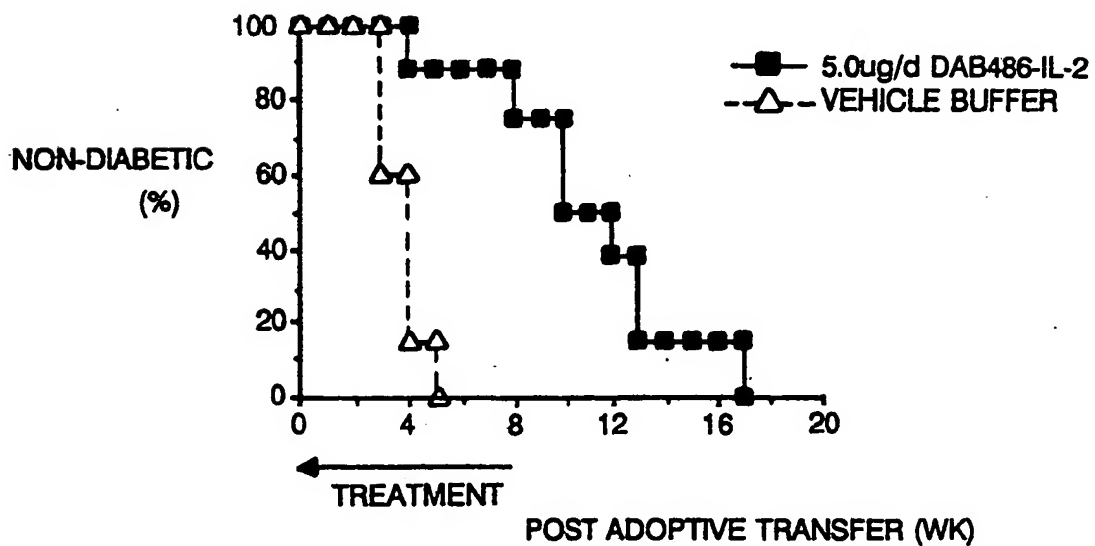


FIG. 1B

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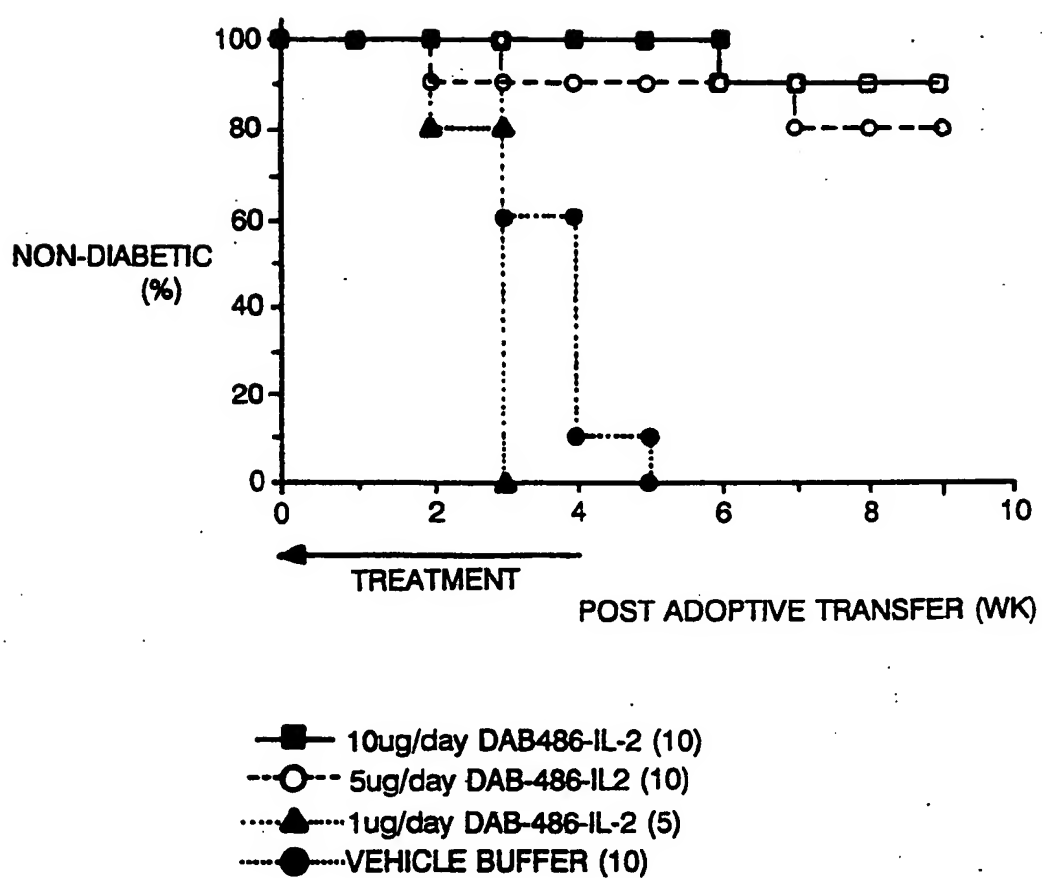


FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01597

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 7/10, 13/00; A61K 45/05, 37/02

US CL :530/350, 324, 402; 514/9; 424/85.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.2; 530/350, 324, 402; 514/9, 866

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IDS from U.S. Application Ser. No. 840,867

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Proceedings of the National Academy of Sciences, Volume 85, No.11, issued June 1988, V.E. Kelley et al., "Interleukin 2-diphtheria toxin fusion protein can abolish cell-mediated immunity <u>in vivo</u> ", pages 3980-3984, entire document.	1.3-5.12 2-5,7,9-11,13
X Y	US, A, 4,675,382 (Murphy) 23 June 1987, entire document.	1.3-5.12 2-5,7,9-11,13

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

03 MAY 1993

Date of mailing of the international search report

19 MAY 1993

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01597

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Current Issues in Pediatric and Adolescent Endocrinology, Volume 37, No. 6, issued December 1990, A.L. Drash et al., "Can Insulin-dependent Diabetes Mellitus Be Cured or Prevented?", pages 1467-1487, especially pages 1475-1476.	2-13
Y	European Journal of Immunology, Volume 20, issued 1990, C.A. Waters et al., "Interleukin-2 receptor-targeted cytotoxicity. Receptor binding requirements for entry of a diphtheria toxin-related interleukin 2 fusion protein into cells", pages 785-791, especially pages 785, 790.	1-2,6,8,10-11



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